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Rapid and Specific Detection of Verotoxin Genes in *Escherichia coli* by the Polymerase Chain Reaction

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A set of four synthetic oligonucleotide probes derived from sequences of the VT1 (Shiga-like toxin I [SLT-I]) and VT2 (SLT-II) genes were used in a polymerase chain reaction (PCR) amplification procedure to detect these genes in some enteric pathogens. A total of 40 verotoxin-producing *Escherichia coli* strains and 43 isolates of other recognized enteric pathogens were studied. PCR amplification products identifying the VT1 and VT2 gene sequences were observed only in nucleic acid extracted from strains found to be VT positive in traditional tissue culture assays. Template nucleic acid extracted from other gram-negative bacteria was found to be negative with the exception of five isolates of *Shigella dysenteriae* type 1 in which good amplification with the VT1 probe was observed. The oligonucleotide probes clearly distinguished VT1 and VT2 strains of *E. coli* and did not give specific amplification with nucleic acid from VTe (a SLT-II variant)-producing *E. coli*. VT1 or VT2 genes or both were not detected in *E. coli* K-12 strain C600 or HB101 or in strains known to express other virulence factors, such as enterotoxins, adhesins, hemolysins, or unrelated cytotoxins. The sensitivity of the PCR procedure for detection of both VT1 and VT2 genes was determined to be 1 ng of total nucleic acid. Furthermore, the VT1 gene was easily detected when only 100 pg of nucleic acid was used as the template in the PCR procedure.

Escherichia coli is among the common bacterial enteric pathogens capable of causing intestinal disease. Several classes of diarrhea-causing *E. coli* are now recognized on the basis of production of virulence factors (6, 17). These bacteria include strains of enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and enteroadherent *E. coli* (17). Strains of an additional *E. coli* group termed verotoxin-producing *E. coli* (VTEC) or Shiga-like toxin (SLT)-producing *E. coli* have been shown to produce bacteriophage-encoded cytotoxins active on Vero and HeLa cells and often include representatives of both EPEC and EHEC. Verotoxigenic *E. coli*, among them serotype O157:H7, have been shown to be closely associated clinically with sporadic and outbreak cases of hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (14, 21, 27). Many VTEC representative of non-O157 serogroups have also been associated with well-characterized intestinal and extraintestinal illnesses, and the clinical significance of these cytotoxin-producing pathogens should not be neglected (14). The VTEC do not harbor enterotoxin genes, and the pathogenesis of this group of virulent *E. coli* may reside in both the capacity to produce cytotoxin(s) and the presence of a unique fimbrial adhesin encoded by a 60-megadalton plasmid (17, 18).

Considering the clinical significance of the VTEC, rapid, specific, and sensitive detection methods are required to identify toxin-producing isolates and replace expensive and time-consuming tissue culture techniques, as reported for enterotoxigenic *E. coli* (23). Three antigenically distinct verotoxins (VT1 [SLT-I], VT2 [SLT-II], and VTe [SLT-II variant]) have been isolated, purified, and partially characterized (14). VTe is produced by strains of *E. coli* responsible for edema disease of swine and is antigenically related to VT2 of EHEC (31). These cytotoxins and the glycolipid Gb_3 receptor for VT1 have been targeted in several enzyme-linked immunosorbent assay techniques of variable sensitivity (1, 15, 24). A cryptic fragment of the 60-megadalton fimbrial adhesin plasmid (EHEC probe) and DNA probes for VT1, VT2, and toxin-converting bacteriophages have been described and used as hybridization probes (6, 13, 22, 27, 30). In the present study, we have designed synthetic oligonucleotide primers for VT1 and VT2 and have demonstrated their specificity and sensitivity in the polymerase chain reaction (PCR) amplification (25) of these gene sequences in clinical and reference cultures of VTEC. The PCR results were compared with results from traditional tissue culture techniques for the identification of specific VT in VTEC associated with human diarrheal disease in Canada.

MATERIALS AND METHODS

Bacterial strains and culture media. A complete list of bacterial strains, including the 40 VTEC used in this study, and their sources appears in Table 1. Cultures designated pEB1, pCG6, HB101, and C600 are derivatives of *E. coli* K-12 strains. pEB1 and pCG6 were maintained on media supplemented with ampicillin. Other bacterial strains were stored on Dorset egg slopes and held in the culture collection of the National Laboratory for Enteric Pathogens, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada. All *E. coli* strains used in these studies were grown in Evans toxin medium (7) for 18 h at 37°C with agitation. Strains of *Aeromonas* spp., *Salmonella* spp., *Shigella* spp., *Klebsiella pneumoniae*, *Hafnia alvei*, and *Citrobacter* spp. were cultured in brain heart infusion broth under similar conditions. Strains of *Campylobacter* spp. were grown for 24 h on Mueller-Hinton agar (Oxoid Ltd., London, England) con-

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TABLE 1. Summary of organisms, cytotoxicity, toxin profiles, and PCR probe result

Culture	Serotype	No. of strains	Cyt[a]	Toxin(s)[b]	Result of PCR amplification	
					VT1 (130 bp)	VT2 (346 bp)
Escherichia coli H19	O26:H11	1	+	VT1	+	−
E. coli	O26:H11	2	+	VT1	+	−
E. coli[c]	O103:H2	1	+	VT1	+	−
E. coli	O111:H8	1	+	VT1	+	−
E. coli[c]	O111:H11	1	+	VT1	+	−
E. coli	O111:H34	1	+	VT1	+	−
E. coli	O111:H−	1	+	VT1	+	−
E. coli	O145:H−	1	+	VT1	+	−
E. coli[c]	O157:H7	1	+	VT1	+	−
E. coli	O126:H8	1	+	VT1, CLDT	+	−
E. coli E32511	O157:H−	1	+	VT2	−	+
E. coli pEB1		1	+	VT2	−	+
E. coli	O113:H21	1	+	VT2	−	+
E. coli	O118:H30	1	+	VT2	−	+
E. coli	O121:H19	1	+	VT2	−	+
E. coli[c]	O132:H−	1	+	VT2	−	+
E. coli	O157:H7	2	+	VT2	−	+
E. coli	O91:H21	1	+	VT2, CLDT	−	+
E. coli	O16:H6	1	+	VT1, VT2	+	+
E. coli	O82:H8	1	+	VT1, VT2	+	+
E. coli	O111:H−	1	+	VT1, VT2	+	+
E. coli	O153:H25	1	+	VT1, VT2	+	+
E. coli	O157:H7	13	+	VT1, VT2	+	+
E. coli[c]	O91:H21	1	+	VT1, VT2, CLDT	+	+
E. coli 412[c]	O139:H1	1	+	VTe[d]	−	−
E. coli pCG6		1	+	VTe	−	−
Shigella dysenteriae 1		5	+	Shiga	+	−
S. dysenteriae 2		1	−[e]	CLDT	−	−
E. coli TD427c2	O25:H−	1	−	LT	−	−
E. coli TD213c2	O128	1	−	ST	−	−
E. coli H10407	O78:H11	1	−	LT, ST	−	−
E. coli HB101		1	−		−	−
E. coli C600		1	−		−	−
E. coli ATCC 25992	O6:H1	1	−		−	−
E. coli[f]	O111:H2	1	−		−	−
E. coli[g]	O124:H−	1	−		−	−
E. coli[g]	O164:H−	1	−		−	−
E. coli	O128:H−	1	−	CLDT	−	−
E. coli	O6:H12	1	−		−	−
E. coli	O55:H4	1	−	CLDT	−	−
E. coli	O55:H10	1	−		−	−
E. coli	O128:H12	1	−		−	−
E. coli[c]	O157:H10	1	−		−	−
E. coli	O157:H16	1	−		−	−
E. coli	O157:H42	1	−		−	−
E. coli	O157:H43	1	−	LT	−	−
E. coli	O22:H43	1	−	HLY[h]	−	−
Aeromonas caviae		1	+	HLY	−	−
A. hydrophila		3	+	HLY	−	−
A. sobria		1	+	HLY	−	−
Campylobacter coli		1	−	CLDT	−	−
C. fetus subsp. *fetus*		1	−	CLDT	−	−
C. jejuni		3	−	CLDT	−	−
C. upsaliensis		1	−	CLDT	−	−
Citrobacter spp.		1	−		−	−
Hafnia alvei		1	−		−	−
Klebsiella pneumoniae		1	−		−	−
Salmonella enteritidis		1	−		−	−
S. hadar		1	−		−	−
S. heidelberg		1	−		−	−
S. typhimurium		1	−		−	−

[a] Cyt, Cytotoxic in Vero monolayers.
[b] LT, Heat-labile enterotoxin; ST, heat-stable enterotoxin.
[c] Nonhuman isolate.
[d] VTe, SLT-II variant associated with edema disease in pigs.
[e] CLDT negative in Vero monolayers, cytotoxic in CHO cell assays.
[f] LA+, Localized adherence in HeLa and HEp-2 cells.
[g] Invasive *E. coli* strain.
[h] HLY, Hemolysin.

TABLE 2. Base sequences, locations, and predicted sizes of amplified products for the VT-specific oligonucleotide primers

Primer	Oligonucleotide sequence (5'-3')	Location within gene[a]	Size of amplified product (base pairs)
VT1a	gaagagtccgtgggattacg	1191–1210	
VT1b	agcgatgcagctattaataa	1301–1320	130
VT2a	ttaaccacacccacggcagt	426–445	
VT2b	gctctggatgcatctctggt	752–771	346

[a] Published gene sequences for VT1 (2) and VT2 (10).

taining 5% sheep erythrocytes under a gas mixture composed of 10% CO_2, 5% O_2, and 85% N_2.

VT and neutralization assays. All culture supernatants to be tested for VT were filtered through 0.22-μm-pore-size cellulose acetate membrane filters (Nalge Co., Rochester, N.Y.). Microcytotoxicity assays (8) were performed by the Vero cell assay system as previously described (16). In brief, serial twofold dilutions of the reference and test filtrates were tested with fresh monolayers of Vero cells. The plates were incubated at 37°C in a 5% CO_2 atmosphere and examined daily for the characteristic cytotoxic effect of VT. Endpoints were determined as the highest dilution (titer) of the toxin-containing filtrate to kill 50% of the monolayer after 48 h of incubation. Quantitative toxin neutralization assays were performed with 5 U of toxin and 20 U of polyclonal anti-VT1 (*E. coli* H19) and anti-VT2 (*E. coli* E32511) as previously described (27).

Enterotoxin and cytotoxin determinations. Heat-labile enterotoxin production was determined in Chinese hamster ovary (CHO) cells (9), mouse adrenal tumor cells (Y-1) (5), and Vero monkey kidney cells (28). Heat-stable enterotoxin was assayed in 3-day-old mice by the standard technique (3). Cytolethal distending toxins (CLDT) were assayed in CHO cells as previously described (11, 12).

Nucleic acid isolation. Nucleic acids were isolated from 5 ml of an 18-h broth culture from all of the bacterial strains listed in Table 1. Cells were pelleted from the cultures at 3,000 rpm for 10 min (IEC Centra 7R centrifuge) and lysed by the standard sodium dodecyl sulfate-lysozyme (sodium dodecyl sulfate, 0.5%; lysozyme, 5 mg/ml) procedure (20). The nucleic acid from all preparations was subsequently extracted with phenol-chloroform and precipitated with ethanol (19). The nucleic acid samples were dissolved in TE buffer (10 mM Tris chloride, 1 mM EDTA [pH 8.0]) and adjusted to a final concentration of 200 μg/ml with PCR buffer (10 mM Tris chloride, 1 mM EDTA, 10 mM NaCl [pH 8.0]).

PCR. DNA samples (1 μg of nucleic acids) were amplified in a 50-μl reaction mixture of the following constitution: 50 mM potassium chloride–10 mM Tris chloride (pH 8.3)–1.5 mM magnesium chloride–0.01% gelatin; 200 μM for each of dATP, dCTP, dGTP, and dTTP; 1 μM for each of the VT-specific oligonucleotide primers described in Table 2 (Oligonucleotide Synthesis Laboratory, Queen's University, Kingston, Ontario, Canada); and 2.5 U of thermostable DNA polymerase (*Taq* polymerase; Perkin Elmer Cetus). The samples were overlaid with 100 μl of mineral oil, denatured for 5 min at 94°C, and subjected to 30 cycles of amplification in a DNA Thermal Cycler (Perkin Elmer Cetus). Parameters for the amplification cycles were denaturation for 2 min at 94°C, annealing of primers for 1 min at 55°C, and primer extension for 1 min at 72°C with autoextension. After the last cycle, the PCR tubes were incubated for 7 min at 72°C. Ten microliters of the reaction mixture was then analyzed by standard submarine gel electrophoresis (2% agarose; 5 V/cm), and the reaction products were visualized by being stained with ethidium bromide (0.5 μg/ml in the running buffer). A reagent blank, which contained all components of the reaction mixture with the exception of template DNA (which was substituted with sterile distilled water), was included in every PCR procedure. To test the sensitivity of the PCR procedure in detecting the VT genes, nucleic acid from an *E. coli* O157:H7 strain was adjusted to a concentration of 200 μg/ml and serial tenfold dilutions were made in PCR buffer.

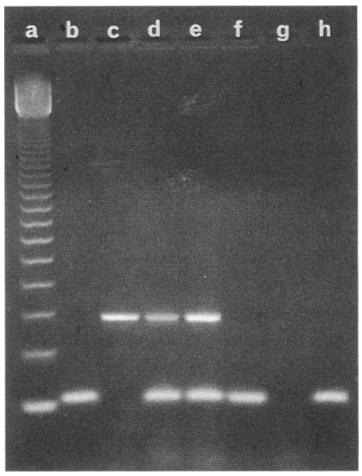

FIG. 1. Occurrence and distribution of PCR amplification products, 130-bp and 346-bp fragments, which specifically detect VT1 and VT2 genes, respectively. Lane a, 123-bp ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.); lane b, *E. coli* H19 (VT1); lane c, *E. coli* E32511 (VT2); lane d, *E. coli* O157:H7, high VT producer (VT1 plus VT2); lane e, *E. coli* O157:H7, low VT producer (VT1 plus VT2); lane f, *E. coli* O111:H8 (VT1); lane g, *E. coli* O6:H12 (VT negative); lane h, *S. dysenteriae* 1 (Shiga toxin).

RESULTS

VT-oligonucleotide probes. Results of primer-directed amplification of the VT1 and VT2 genes are represented in Fig. 1 and 2. Two pairs of oligonucleotide primers, VT1 and VT2 (Table 2), were used in the PCR. The primers were designed on published sequences for the VT1 (2) and VT2 (10) genes (4). The VT1 primers delineated a 130-base-pair (bp) fragment of the gene coding for the B subunit of the toxin, and the VT2 primers targeted a 346-bp fragment in the region coding for the A subunit. Figure 1 shows the presence and distribution of the two amplified products when DNAs from representative VTEC strains of *E. coli* were used as a template. The sizes of the amplified products were as predicted from the design of the primers, that is, 130 bp for the VT1 primers and 346 bp for the VT2 primers.

Specificity of oligonucleotide probes for detection of VT1 and VT2. Well-characterized VTEC strains from the National Laboratory for Enteric Pathogens culture collection were used to evaluate the specificity of the VT1 and VT2 oligonucleotide probes described in Table 2. The VT-producing strains are listed in Table 1 with the corresponding toxin profile determined by Vero cell tissue culture assays and the observed PCR amplification products. A total of 18 EHEC, 10 EPEC, and 2 VTe isolates were included in the 40 VTEC strains tested. Genetically engineered strains of VT2

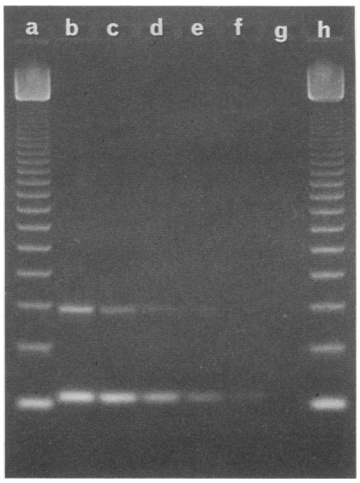

FIG. 2. Sensitivity of the PCR protocol in detecting VT1 and VT2 genes in *E. coli* O157:H7. Lane a, 123-bp ladder; lane b, 1 μg of template; lane c, 100 ng of template; lane d, 10 ng of template; lane e, 1 ng of template; lane f, 100 pg of template; lane g, 10 pg of template; lane h, 123-bp ladder.

(pEB1) and VTe (pCG6) were tested in addition to the corresponding human and porcine isolates. *E. coli* K-12 strains (HB101 and C600) and a collection of recognized enteric pathogens were used to determine which of these strains carried the VT1 or VT2 genes by both the PCR and standard tissue culture procedures.

In this study, the identification of bacterial strains as VT1, VT2, both VT1 and VT2, or non-VT producers by the PCR protocol corresponded without exception with the type of VT activity as determined by toxin-specific antibody neutralization in Vero cell assays. Both the *E. coli* K-12 strains (C600 and HB101) and other enteric pathogens tested were found not to possess structural gene sequences coding for either VT1 or VT2. No amplification products specific for the genes responsible for VT production were detected in DNA from reference toxigenic, invasive, adherent, and hemolytic strains of *E. coli*. In view of the extensive homology of Shiga toxin and VT1 (>99%) (29), it was expected that *S. dysenteriae* 1 strains positive for Shiga toxin would show amplification with the VT1 primers (Fig. 1, lane h). One *E. coli* O6:H12 originally documented as VT positive in 1983 was found to be both toxin and probe negative in the present study (Fig. 1, lane g).

Sensitivity of VT1 and VT2 gene detection by PCR. All strains designated VT positive in Vero cell assays were found to harbor one or both of the VT gene sequences detected in the PCR reaction. In the VTEC strain collection used in this study, titers of extracellular VT determined by Vero cell assays varied from 1:8 to 1:2,048. Strains with poor expression of VT genes (titers ≤1:16) were consistently found to be probe positive with normal amplification of VT1 or VT2 gene sequences or both. There was no correlation between titers of VT and sensitivity of the PCR in detecting and identifying the appropriate gene (Fig. 1, lanes d and e). VT1 and VT2 genes have been identified in a low-producing (titer = 1:64) *E. coli* O157:H7 strain when only 1 ng of total nucleic acid was used as a template in the PCR assay (Fig. 2). Furthermore, the amplified fragment specific for the VT1 gene was clearly detected when the nucleic acid template used in the PCR was further diluted 10-fold to 100 pg.

DISCUSSION

VTEC have been very closely associated with hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (14, 17, 21). Although *E. coli* serotype O157:H7 has been the most thoroughly linked with these diseases in humans, other non-O157 VTEC have been associated with these and other enteric diarrheal diseases (14, 27). Improved identification of verotoxigenic strains in clinical samples is required in order to further elucidate the association of VTEC with diseases of adults and children. The PCR technology will allow large-scale screening of many colonies from clinical material or contaminated foods.

The oligonucleotide probes demonstrated a high specificity in that all VT-producing isolates were detected, including EHEC and other VTEC. The probes were clearly able to distinguish VT1, VT2, and VT1- and VT2-producing strains and did not show specific amplification with DNA from VTe-producing *E. coli*. Nucleic acid from reference enterotoxigenic *E. coli*, enteroinvasive *E. coli*, nontoxigenic EPEC, and enteroadherent strains or two K-12 strains of *E. coli* did not show amplification of the requisite fragments for the VT1 or VT2 genes in this PCR protocol. In view of these results, the role of low-level Shiga-like toxin production in K-12 strains and some EPEC serotypes as previously described (21) must be carefully reevaluated. Further, DNA from CLDT-producing and hemolytic *E. coli* strains were probe negative. On occasion, weak amplification of some nonspecific sequences outside the size range of the predicted products was observed. This weak amplification is not unexpected in the PCR technique if the target sequence is absent initially or present in extremely low quantities (25, 26). No primer-specific amplification was detected when the DNA template was isolated from VT-negative *E. coli* or from other strains of enteric pathogens tested, which included *Aeromonas* spp., *Campylobacter* spp., *Salmonella* spp., *Citrobacter* spp., *K. pneumoniae*, *H. alvei*, and *S. dysenteriae* 2.

In the present study, no cultures were VT positive by the Vero cell assay and VT negative by probe. The oligonucleotide probes are specific for the two toxin genes which were readily detected in several VTEC serotypes from human and nonhuman sources. Although gene expression varied considerably, as evidenced by the wide range of extracellular toxin titers, we observed an excellent correlation of probe detection of VT1 and VT2 genes and the bioassays when a well-defined phenotypic group of VTEC was investigated. The oligonucleotide primers for the VT2 gene were designed to avoid areas of sequence homology with the gene for VTe (31). Hence, these PCR primers differentiate the VT2- and VTe-producing strains of *E. coli* and allow definitive identification of the VTEC gene products. The significance of VTe in human disease, if any, has not been established (14).

This study was aimed at establishing the specificity and sensitivity of the VT1 and VT2 primers in the PCR protocol. Published reports which have addressed the level of detection of specific gene sequences in PCR protocols have demonstrated a level of sensitivity greater than that demonstrated by other established procedures (24a, 32). In this study, we have been able to easily detect the VT1 or VT2 genes or both when as little as 1 ng of total nucleic acid was used as a template in the PCR assay.

Epidemiological studies to determine the scope and significance of VTEC disease have been limited by the lack of simple and rapid assays, since the tissue culture assays are expensive and time consuming. Enzyme-linked immunosor-

bent assay systems have been developed to detect VT and Shiga toxins (15, 24), but major drawbacks with these systems are related to specificity and sensitivity. A recently described receptor-enzyme-linked immunosorbent assay for VT1 is very sensitive but detects only VT1 (1). The PCR technique that we describe is relatively simple and, combined with its superior sensitivity and specificity, should readily enable rapid screening of many colonies per sample. Subsequent to extraction of DNA, the PCR protocol yields in 4 h data which require several days by traditional tissue culture assays. Mixed VTEC infections could be easily detected, and extensive data could be generated concerning the distribution of VTEC and, particularly, non-O157 VT-positive *E. coli* strains in the pathogenesis of hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura.

The PCR test for amplification of VT1 and VT2 gene sequences has demonstrated universality in that both toxin genes can be detected and differentiated across a wide spectrum of verotoxigenic *E. coli*. With the growing numbers of verotoxigenic *E. coli* serotypes other than O157:H7, which presently exceed 50 (14), which have been associated with hemorrhagic colitis or hemolytic uremic syndrome or both and for which no biochemical markers or diagnostic antisera are currently available, the PCR protocol we have described here permits rapid, accurate, and inexpensive detection of these important toxin genes in clinical material and possibly in contaminated foods.

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